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Higher survival but smaller size of juvenile Dungeness crab (*Metacarcinus magister*) in high CO_2

Paul McElhany [a,*], D. Shallin Busch [a], Amanda Lawrence [b], Michael Maher [a], Danielle Perez [a], Emma M. Reinhardt [c], Kate Rovinski [a], Erin M. Tully [d]

[a] Conservation Biology Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Ocean and Atmospheric Adminstration, 2725 Montlake Blvd. E., Seattle, WA, USA
[b] National Sea Grant College Program, 1315 East-West Highway, Silver Spring, MD 20910, USA
[c] University of North Carolina at Chapel Hill, Wilson Hall, 110 South Road, Chapel Hill, NC 27599, USA
[d] Oregon State University, Burt Hall 236, 2651 SW Orchard Ave, Corvallis, OR 97331, USA

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ABSTRACT

Dungeness crab (*Metacarcinus magister*) are the most valuable fishery on the U.S. West Coast and both larval and adult Dungeness crabs are important components of regional food webs. Previous experiments have shown decreased survival and a slower development rate for Dungeness crab zoea reared in water with high CO_2, indicating a susceptibility to ocean acidification. In this study we reared late-stage megalopae and juvenile Dungeness crabs in both ambient and high CO_2 conditions for over 300 days. Counter to expectations, crabs reared in high CO_2 had a higher survival rate than those reared in ambient conditions and crabs in high CO_2 transitioned more quickly in one of the stages (J5 to J6). However, crabs reared in high CO_2 were generally smaller and had a higher resting metabolic rate than crabs in ambient CO_2. We hypothesized that two separate mechanisms were in effect, with one process driving survival and a second process driving size and respiration rate. We further hypothesized that increased mortality in ambient CO_2 could be caused by a CO_2-sensitive microbial pathogen, but that size and respiration differences were caused by the direct effects of CO_2 on the crabs themselves. Overall, the zoea stages seem more sensitive to CO_2 than the megalopae and juvenile stages.

1. Introduction

Experiments have shown that many crab species (infraorder Brachyura) are sensitive to elevated seawater CO_2 concentrations and are potentially vulnerable to ocean acidification (OA). Effects observed in experiments that rear crabs in high CO_2 include changes in survival, growth rate, metabolic rate, behavior, internal acid-base balance, exoskeleton structure and molecular pathways (Ceballos-Osuna et al., 2013; Dickinson et al., 2021; Gravinese et al., 2019; Long et al., 2020; Miller et al., 2016; Trigg et al., 2019). Not all crabs exhibit CO_2 sensitivity, with hypotheses about species differences centered on life history characteristics (Carter et al., 2013; Hu et al., 2016; Pane and Barry, 2007; Shaughnessy et al., 2017; Spicer et al., 2007; Turner, 2016). Early stages of marine organisms are hypothesized to be more vulnerable to OA (Kurihara, 2008). However, data on crabs suggest that the life-stage sensitivity can be species-specific (Noisette et al., 2021; Small et al., 2015; Small et al., 2016).

Dungeness crab (*Metacarcinus magister*), which range from the intertidal to ~150 m depth from Central California to the Aleutian Islands, support the most valuable fishery on the U.S. West Coast with an ex-vessel value in 2019 of ~$200 M (National Marine Fisheries Service, 2021) and a current population abundance that is stable or slightly increasing (Richerson et al., 2020). After hatching from eggs attached to the mother's abdomen, Dungeness crab larvae progress through five planktonic zoeal stages, then molt to the transitional megalopae stage before settling to the seafloor and molting to the juvenile stage. Juvenile Dungeness crabs undergo multiple molting events as they grow in size to reach maturity after two years. Settling megalopae and young-of-the-year (YOY) juvenile Dungeness crab prefer substate habitats with bivalve shells and/or eel grass, which provide more cover from predators compared to open mud flats (Fernandez et al., 1993). Predators on juvenile Dungeness crab include fish (e.g., sculpins) and larger Dungeness crab (Fernández, 1999). Massive juvenile crab recruitment events have been observed, leading to suggestions that cannibalism plays an

* Corresponding author.
E-mail address: paul.mcelhany@noaa.gov (P. McElhany).

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important role in density-dependent population dynamics (Galloway et al., 2017). Although YOY Dungeness crab primarily eat small bivalves and crustaceans, they are opportunistic carnivorous scavengers and will even consume epiphytic diatoms (Jensen and Asplen, 1998).

Previous experiments have shown that when reared in high CO_2 seawater at concentrations expected under future OA conditions Dungeness crab eggs hatch more slowly and zoea have lower survival and slower development rates (Miller et al., 2016). Field correlations also suggest Dungeness crab larvae are sensitive to high CO_2. Dungeness crab megalopae collected from sites with higher $\Delta\Omega_{cal,60}$ exhibited different exoskeleton characteristics from those at lower $\Delta\Omega_{cal,60}$, where $\Delta\Omega_{cal,60}$ is defined as the integrated difference between the calcite saturation state at the surface and at 60 m depth (Bednarsek et al., 2020). Based on this observation, Dungeness crab megalopae exoskeleton condition has been suggested as a possible biological indicator of OA.

This study reports on the effect of elevated CO_2 on late-stage megalopae and first-year juvenile Dungeness crab – two stages which have not been evaluated before in a controlled experimental setting. The goals were to inform assessments of the overall vulnerability of Dungeness crab to OA and begin laboratory testing of megalopae sensitivity, information needed to evaluate their utility as a biological indicator. The Dungeness crabs used in this study were collected from the Salish Sea, a fjordal estuary system in the NE Pacific, a region with naturally high CO_2 levels that is considered especially at risk from anthropogenic acidification (Bianucci et al., 2018; Cai et al., 2020; Feely et al., 2010; Lowe et al., 2019; Pelletier et al., 2018; Reum et al., 2014). The mean pH in the Puget Sound region of the Salish Sea is currently ~7.8 (the ambient pH treatment in this experiment) and the pH in some parts of the Salish Sea may occasionally drop below 7.2 (the low pH treatment in this experiment). Although, a pH of 7.2 does occasionally occur in Puget Sound and pH values that low are expected to be more common in the future (Khangaonkar et al., 2019), the treatment level of 7.2 was selected to represent the lower range of expected pH to reveal any CO_2 sensitivity in the exposed life stages. If sensitivity were observed at pH 7.2, further investigation would be warranted; however, if no sensitivity were observed at pH 7.2, it is unlikely that OA would have an impact on the measured traits. Because of the conditions driving naturally low pH, waters in the Salish Sea are often undersaturated with respect to calcium carbonate with the frequency and spatial extent of undersaturation expected to increase with OA.

2. Materials and methods

2.1. Crab collection

Live Dungeness crab megalopae were collected by the Lummi Nation Department of Natural Resources using light traps over the night of August 16–17, 2018 at two locations in the Salish Sea near Lummi Island in North Puget Sound (Hale's Pass at 48.72923°, −122.667° and Sandy Point at 48.81622°, −122.711°; supplemental Fig. 1; see Cook et al., 2018 for discussion of light trapping in Puget Sound). After retrieval of the light traps, the crab were transported to the Lummi Tribal Center in Bellingham, Washington. The light traps, which are part of a separate Lummi Nation Dungeness megalopae monitoring project, were constructed from square 20 L buckets with four inlet funnels and a battery powered LED light source (supplemental Fig. 2). Several hundred megalopae from the two sites were separated from bycatch and transported in coolers with ice packs and air bubblers from the Lummi Tribal Center to the NOAA Northwest Fisheries Science Center Mukilteo Research Station in Mukilteo, Washington (supplemental Fig. 1).

2.2. Megalopae rearing

On August 17, equal numbers of megalopae from each site were pooled, then distributed into the wells of 6-well plastic culture plates with one megalopae per well. Well plates containing 222 megalopae were distributed into six "CO_2-chambers" (Fig. 1), three of which were designated as "ambient CO_2" ($n = 99$) and three as "high CO_2" ($n = 123$). The CO_2-chambers are modified refrigerators that 1) regulate temperature using high-precision thermal sensors with a custom control system, 2) control atmospheric CO_2 concentration using mass flow controllers, and 3) control the temporal pattern, colour and intensity of light using 4-colour LED strips (supplemental Fig. 3). All chambers were held at 12 °C with the ambient CO_2-chambers maintained at a CO_2 concentration of 400 ppm and the high CO_2-chambers at 2800 ppm. Water to fill the well plates (5 mL per well) was taken from 2 L jars of 1 μm filtered and U.V. treated seawater from Puget Sound that had been equilibrated to each chamber's CO_2 concentration and temperature by bubbling mixed-gas from the chamber's mass flow controllers inside the chamber for ~1 h. The megalopae were checked for survival and molting every day except the first two days after placing them in CO_2-chambers when logistical constraints prevented observation. No crabs were observed to have died by the first observation period; however, ~40% of the megalopae molted to juveniles by the first observation period and the delay in first observation created uncertainty about whether the megalopae molted the first or second day in treatment. In the analysis, megalopae were assigned to have molted on the first observation day. To test the sensitivity to this assumption, we re-ran the survival and development rate analysis (described below) assuming either all of the ambiguous megalopae molted the first day or the second using a Monte-Carlo approach to randomly assign molt date to ambiguous megalopae. We found no effect of molt date uncertainty on treatment effect results. Megalopae were transferred to new well plates with a new batch of equilibrated seawater every three days using a small spoon. The megalopae were fed newly hatched *Artemia* naupli (San Francisco Bay brand) ad libitum when initially placed into well plates and after each well plate transfer (i.e. every three days). As soon as a megalopae was observed to have molted, it was transferred to the juvenile rearing system.

2.3. Juvenile rearing

Juveniles were reared in individual 250 mL polypropylene screw-top mesh jars that had 5–10 mm of silica sand in the bottom (supplemental Fig. 4). The silica sand provided cover habitat and potentially helped keep the exoskeleton free of parasites (Pam Jensen, pers. comm.). The jars were placed in polycarbonate holders that maintained them just below the surface in a 1.2 m diameter, 700 L flow through seawater tanks (supplemental Fig. 4). Tank seawater was pumped from a Puget Sound intake 10 m deep and ~ 30 m from shore and passed through a sand filter. Tanks were maintained at ambient temperature and each tank had a flow rate ~ 15 L per minute. Half of the tanks (3) were maintained at ambient pH (mean = 7.77), and pH in the other three tanks was controlled at a target pH of 7.2 using a Durafet® pH sensor feed-back system that regulated the injection of 200 microsecond bursts of pure CO_2 into the tank though air stones near the bottom of the tank. The exact frequency of CO_2 injection depended on the pH and temperature of incoming water, but was approximately every 90 s. The ambient CO_2 varied with the incoming seawater pumped into the station, whereas the high CO_2 treatment was maintained at a constant pH. It would have been preferable to have both treatments either with constant pH or to have the high CO_2 treatment as an offset of the ambient conditions, but neither of these options were implemented because of technical limitations with the experimental system.

Juveniles from megalopae in high CO_2-chambers were placed randomly in one of the three low pH tanks and juveniles from megalopae in the low CO_2-chambers were placed randomly in one of the ambient pH tanks. The juveniles were checked every day for survival and molting. The crabs in all treatments were fed ad libitum twice per week the same diet consisting primarily of small pieces of market squid (*Doryteuthis opalescens*), with occasional geoduck (*Panopea generosa*) or salmon (*Oncorhynchus kisutch*); any uneaten food was removed before

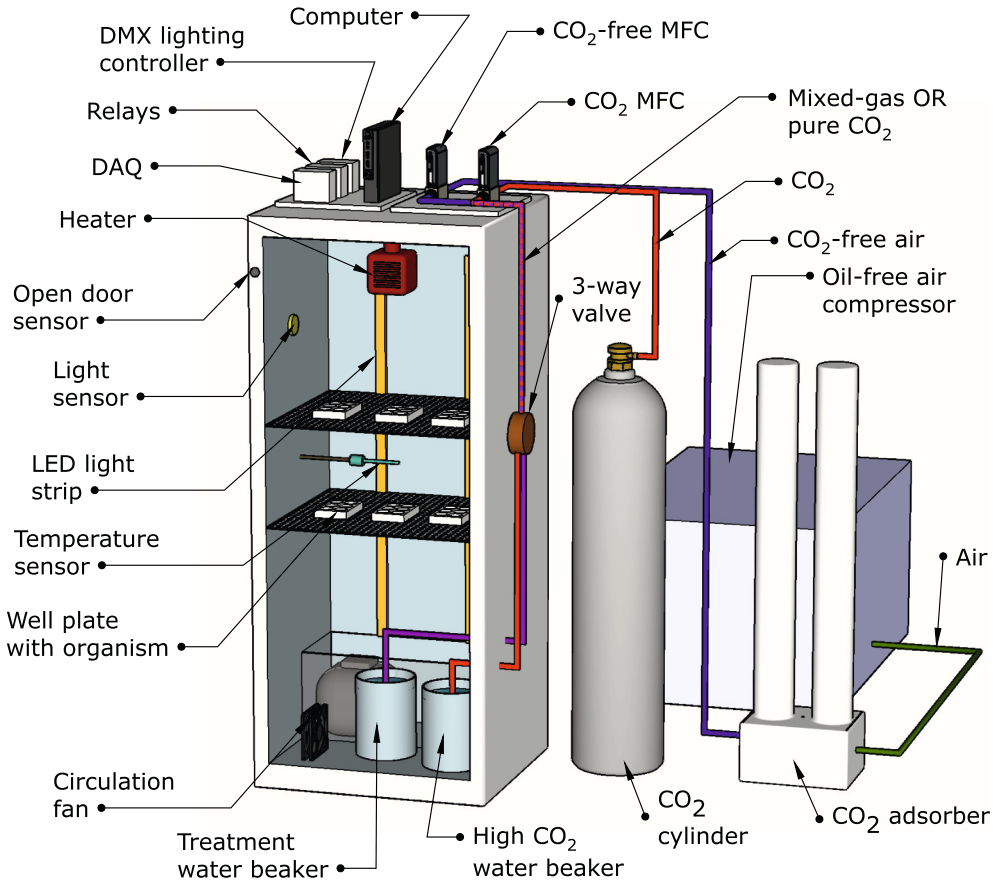


Fig. 1. Simplified schematic of a CO_2-chamber used to rear Dungeness crab megalopae in individual well plates. The CO_2-chambers control temperature, CO_2 concentration, and light colour, temporal pattern and intensity. See supplemental fig. 2 for more details on CO_2-chamber operation.

new food was added. As the nutritional needs of growing Dungeness crab are unknown, we considered some variation in the diet as a prudent way of providing required micronutrients. Fresh sand was added as needed to replace that which was slowly lost through the mesh jars. The tanks were drained and cleaned once per week. All of the crabs from one tank were transferred to another tank with the same treatment conditions once per week, rotating through all three tanks, in an effort to reduce long-term tank effects. Thus, since the crabs were moved from tank to tank as a group, "tank group" is a random factor in the statistical analysis. The experiment ended on July 10, 2019, a total of 327 days after megalopae were placed into CO_2-chambers. On July 11 and 12 all surviving juvenile crabs were flash frozen at −80 °C for future genetic and chemical analysis.

2.4. Seawater chemistry

Mass flow controller output was recorded to provide a continuous measurement of the CO_2 concentration input into each chamber. Spectrophotometric pH measurements were taken of the CO_2-equilibrated water used to fill the wells in each chamber on each water-change day. In the juvenile tanks, pH values from the tris buffer calibrated Durafet® probes were recorded daily and spectrophotometric pH measurement taken weekly. Spectrophotometric pH measurements followed the best practices (Dickson et al., 2007) using an Ocean Optics USB 2302000+ spectrophotometer. Salinity of water in the CO_2-chambers and juvenile tanks was recorded using a hand held salinity meter (Orion Star A322) and, starting on November 20, 2018, salinity in the juvenile tanks was recorded daily from a continuous conductivity sensor (Analytical Technology, Inc. Q46CT) located in one of the tanks. The ATI salinity readings were validated with occasional measurements in all of the tanks with the handheld sensor. Temperature of the CO_2-chambers was recorded continuously using the chamber's temperature probe (Labjack EI-1034) and verified periodically using a reference thermometer (Fluke model 1523). Temperature measurements from the Durafet® pH probes in the juvenile tanks were recorded once per day, with occasional validation using the reference thermometer. Alkalinity in the chamber water and in the juvenile tanks was calculated using a Mukilteo-specific alkalinity/salinity relation as described in (Trigg et al., 2019). The Mukilteo relationship ((Alk = 50.345 * salinity +522.506) has a similar slope to the Washington-wide equation developed by (Fassbender et al., 2017), but with an offset intercept, likely reflecting the influence of local rivers.

2.5. Juvenile size analysis

Several days after each molting (mean = 15d), after which the exoskeleton was assumed to have completely expanded, the live juveniles were photographed under a dissecting microscope for size analysis. Live crabs were dabbed dry before being placed in a six-well plate, then photographed using Infinity Analyze software (Teledyne Luminara) combined with a Nikon SMZ 745 T microscope. Magnification varied with the size of the crab so that all crabs could be photographed at near full frame. After photographing, juveniles were immediately returned to treatment tanks. Crab width (distance along the transverse axis of the carapace) was measured using the Infinity Analyze software at the time the photo was taken. Crab length (distance along the sagittal axis of the carapace) was measured from the photographs at a later date using the ImageJ software straight-line tool (Rueden et al., 2017). Discarded exoskeletons after molting were also collected for size analysis to determine whether they could be used as a size metric in future

experiments that is more convenient than photographs of live animals. The molted exoskeletons were air dried, then photographed after molting. The discarded exoskeletons from the crabs in J1-J3 stages were photographed a mean of 13d after molting and the exoskeletons from the crabs J4-J6 stages were photographed a mean of 78d after molting (a U.S. government shutdown delayed photographing molts). The legs of the discarded exoskeletons were removed to obtain a clear view that was orthogonal to the plane of the carapace.

2.6. Respirometry

Between June 4 and June 27, 2019, the base metabolic rate of a subset of the stage 5, 6 and 7 juvenile crabs was measured by recording the rate of oxygen consumption in a sealed chamber (sample sizes: J5 = 10, J6 = 40, J7 = 19). Food was removed or withheld 24 h before a respirometry trial to reduce the influence of transient digestion activity. Crabs were dabbed dry, then weighed prior to the trial so that the respiration rate could be standardized by crab weight. In a respirometry trial, individual crabs were placed in custom acrylic chambers with an internal volume of 516 mL. The chambers had an internal stir bar system that circulated water during the trials. Chambers were filled with water from the same treatment conditions in which the crab was reared (i.e., crabs from ambient treatment in ambient pH water and crabs from low pH treatment in low pH water). The crabs were inserted in the chambers and the chambers sealed while submerged in the treatment water to prevent air bubbles. Oxygen inside the chamber was measured continuously using a PreSens oxygen measurement system (OXY-10). Six or seven respirometry chambers were run simultaneously and one chamber in each trial set was run as a "blank" (i.e., no crab present) to measure the base-line respiration of microorganisms present in the seawater. The chambers were placed in a water bath with flowing water at ambient temperature (mean 11.1 °C). For analysis, the first 30 min that the crab was in the chamber was considered the initial acclimation period while the crab settled into the surroundings and oxygen rate was evaluated over the next two-hour period (except for one trial, in which only one hour of data were recorded because of instrument malfunction). At the end of the trial, the dissolved oxygen saturation level in the chambers was >80% and crabs were not considered to ever experience low oxygen stress. After the trial, crabs were returned to treatment tanks.

2.7. Survival and molting statistics

Analysis was conducted using the R statistical package (version 4.0.0; (R Core Team, 2020). The effect of pH on overall survival was evaluated using a hazard model, with CO_2-chamber and tank group considered random variables (i.e., clustered frailty terms) in a cluster randomized trial design (Brown et al., 2015; Cai and Shen, 2000).

The transition hazard function (i.e., instantaneous transition rate at time *t*, given that the transition has not happened prior to *t)* is:

$$\lambda(t) = z_i \lambda_0 e^{\beta^T x}$$

Where $\lambda(t)$ is the hazard at time *t*, $\lambda_0(t)$ is the baseline hazard, z_i is a cluster-specific term modifying the baseline hazard in group *i*, β is a vector of the transition-specific treatment coefficients and *x* is the vector of covariates (i.e., treatment values). The hazard equation was fit using the frailtyEM R package (Balan and Putter, 2019, 2020). The distribution of z was selected from options of gamma, positive stable, inverse gaussian, and non-central gamma by comparing likelihood values. The decision on whether to include the frailty term at all or opt for a simpler cox proportional hazard model was made based on the Commenges-Andersen test for heterogeneity of frailty terms and by likelihood ratio test comparing the model with and without frailty.

A multi-state Markov hazards model was used to evaluate the effect of pH on molting rate and stage-specific survival. In the multi-state model, crabs at each molt stage could either molt to the next stage or die, resulting in 15 possible transitions (supplemental fig. 5). The frailty hazard model is as described in eq. 1, but with a separate hazard function $\lambda_q(t)$ and baseline hazard $\lambda_{q0}(t)$ for each *q* transition (i.e., separate strata for each transition) (Putter et al., 2007; Therneau et al., 2020).

The data were reformatted into a form appropriate for a multi-state model using the mstate R package (de Wreede et al., 2010, 2011), and the hazard coefficients were fit using the coxme package (Therneau, 2020). The coxme model assumes a log normal distribution for the mixed effects term, z_i, and fits the coefficients using a proportional hazards partial likelihood approach. A permutation test was applied to evaluate whether the transition-specific β values significantly differ from zero. The permutation test was selected as more robust than a distribution-based test given the relatively low number of tank groups (3 per treatment) and the potential for type-1 errors in cluster randomized trials with few clusters (Cai and Shen, 2000; Leyrat et al., 2018). To further reduce the risk of type-1 errors, a Holm-Bonferoni correction was applied to account for testing 15 separate transitions. For comparison, a simple, cox proportional hazard model without the frailty term (i.e., CO2-chamber/tank group effect) was also evaluated. For visualization, probability plots of molt stage and survival for ambient and low pH treatments were generated using the mstate R package. The mstate package is not compatible with mixed effects models so the probability plots were generated with a model containing only pH treatment as a factor. The plot presents the marginal effect of CO_2 treatment ignoring the CO_2-chamber and tank group random variables.

2.8. Size statistics

Analysis was conducted using the R statistical package (version 4.0.0)(R Core Team, 2020). Size response metrics included the carapace length and width measurements, the ratio of length to width (which provides a measure of "roundness"), the weights collected as part of the respirometry analysis, and the weight to width ratio (which provides a metric related to density). For the width, length and length/width metrics, the overall effect of CO_2 treatment was evaluated as a repeated-measures (mixed-effects) model to account for measurements taken on the same individual at each life-stage. Mixed effects were analyzed using the lme4 R package. Considering potential CO_2-by-stage interactions and random tank effects, the full model is:

$$\text{size\_metric} \sim CO_2 + \text{stage} + CO_2{}^{*}\ \text{stage} + \text{random}(\text{crab}) + \text{random}(\text{tank\_group})$$

Likelihood ratio tests using the R stats and RLRsim packages were used to select among the potential models with all combinations of including or excluding the interaction and tank group terms. Although stage is an ordinal variable, it was treated as an interval variable in this analysis, which implies an equal weight of each stage on size. The weight measurements were taken only once per individual so the full model was simply.

$$\text{weight\_metric} \sim CO_2 + \text{stage} + CO_2{}^{*}\ \text{stage}$$

Weight measurements were taken from every tank group, but sample sizes were too small to evaluate any tank group effects (Supplemental table 2). Likelihood ratio tests were used to select among weight models with and without the interaction terms. Life stage was treated as an ordered categorical variable and entered in the model using polynomial contrasts. In addition to the overall analyses, treatment comparisons were made separately for each metric for each stage. Models with and without the random tank effect term were compared by likelihood ratio test.

Carapace width was also modeled as a function of the day since the crab molted to a juvenile to consider overall trajectories of size independent of stage. Size was modeled as repeated measures (mixed effects) evaluating both linear and quadratic models to capture non-linearities in the relationship. AIC values were used for model selection. Because the

growth data did not show an inflection point and analyses were conducted on length rather than weight, a simple empirical quadratic model was evaluated rather than more assumption-laden, weight-based growth models such as the Gompertz and von Bertalanffy.

$$\text{width} \sim \left(\text{day} + \text{day}^2\right)^* \text{CO}_2 + \text{random(crab)}$$

2.9. Respirometry statistics

Analysis was conducted using the R statistical package (version 4.0.0) (R Core Team, 2020). The respiration rate for each crab was corrected for ambient seawater respiration by subtracting the respiration for the blank (no crab) respirometry chamber in each trail from the crabs evaluated in that trial. Respiration rate was analyzed as a family of simple linear models that included combinations of treatment, stage, weight and interaction terms as predictor variables. The linear relationship between respiration rate and weight was also estimated separately for ambient and high CO_2 treatments.

3. Results

3.1. Seawater chemistry

Temperature, ambient pH and salinity varied seasonally in the juvenile exposure tanks (Fig. 2; supplemental table 1). The high monthly mean temperature of 12.8 °C occurred in September 2018 with the low mean of 8.3 °C in March of 2019. Salinity means varied from 29.4 psu in April to 30.8 psu in October, with calculated alkalinity means in the same months of 2002 μmol kg^{-1} and 2073 μmol kg^{-1}, respectively. The drop in salinity observed in January likely reflects a winter increase in freshwater river input that is typically observed in this part of Puget Sound (supplemental Fig. 6). Ambient pH increased approximately linearly during the course of the experiment with an initial mean in August 2018 of 7.68 and a final mean of July, 2019 of 7.80. The minimum and maximum monthly mean pH values were 7.68 in August 2018 and 7.85 in June, 2019. Changes in ambient pH reflect both variation in the natural water of Puget Sound and potential biological activity in the plumbing of the facility's seawater intake, filtering and storage system. The high-CO_2 treatment pH was under feed-back control and maintained a constant mean throughout the experiment of 7.19 (mean pCO2 = 3055 μatm). The calcite saturation state of the ambient treatment remained above one throughout the experiment with an overall mean value of 1.55. The high-CO_2 treatment was consistently undersaturated with an overall mean calcite saturation state of 0.43.

3.2. Survival and molting rate

Megalopae had high survival (96.0% survival in ambient CO_2 and 97.6% survival in high CO_2) with no statistically significant effect of CO_2 treatment (hazard model with inverse Gaussian frailty, $p = 0.25$). The megalopae molted to the J1 stage relatively quickly after being placed in the CO_2 chambers (mean = 4.3 d). Two megalopae from the high CO_2 treatment that had not molted after 10 d were removed from the experiment and treated as right censored observations in the survival analysis. The number of individuals at the start of each stage for the two CO_2 treatments is shown in Table 1.

The overall survival of juveniles reared in high CO_2 was higher than those reared in ambient CO_2 (Fig. 3). The final fraction of survivors at the end of the experiment was 18.9% (18/95) in ambient CO_2 and 34.7% (41/118) in high CO_2. In the hazard model, the frailty term (tank group) was not significant (Commenges-Andersen $p = 0.763$, LRT $p = 0.493$), so the analysis was conducted as a cox proportional hazards model with only treatment as a fixed effect ($p = 0.0005$). During the experiment, the odds of dying were over twice as high in low CO_2 as in high CO_2 (exp(β) = 2.22). During the experiment, juveniles would occasionally escape when the containers were open during feeding or they escaped through small joints in the mesh jars. These escapees were treated as right censored observations (as were all crabs still alive at the end of the experiment). For comparison, the analysis was run both with and without the inclusion of the right censored escapees in the dataset, which produced identical results with regard to treatment significance.

The probability of being in a particular stage as a function of time is shown in Fig. 4. In considering life-stage specific survival transitions (Fig. 5), only the J1 to J2 transition was found to be significant, with the odds of dying over four times greater in low CO_2 than in high CO_2 (coxme permutation model, $p = 0.006$, exp.(β) = 4.79). Because none of the four crabs from the ambient CO_2 treatment that made it to the J7 stage died before the end of the experiment, it was not possible to make any inference from the hazard model with respect to J7 survival. With a sample size of only four crabs in one of the treatments, no attempt was made at any other sort of analysis of J7 survival and it is considered unknown.

In the molting transitions (Fig. 6), only the J5 to J6 transition was significantly different among treatments, with the odds of transitioning over three times greater for crabs in high CO_2 compared to crabs in low CO_2 (coxme permutation model, $p \leq 0.0001$, exp.(β) = 3.125). The mean duration of the J5 stage was 8.6 days shorter for crabs reared in high CO_2 compared to those reared in ambient conditions.

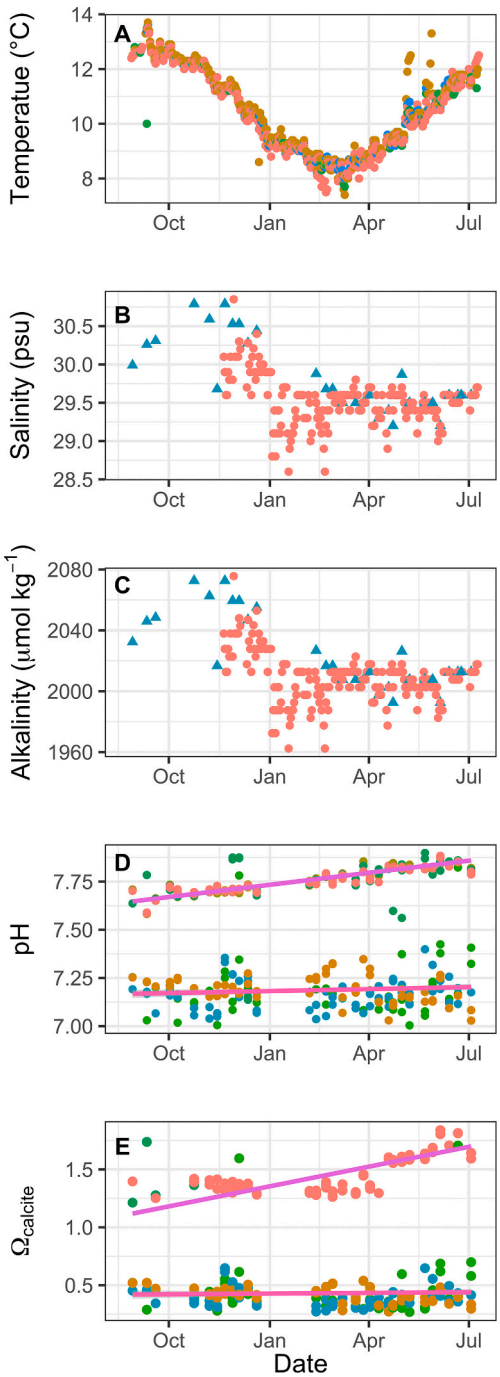
3.3. Size

Juvenile Dungeness crabs reared in ambient CO_2 were overall larger at a given stage and had a wider shape than crab reared in high CO_2, though there was no difference in weight (Fig. 7, supplemental Table 2). The width and length/width models include both a main treatment term and a treatment by stage interaction term, with only the interaction being significant (Table 2). This is consistent with the stage-level analysis, which shows some stages with substantial treatment differences in size but not others (Fig. 7, supplemental Table 2). For the stage-level analysis, the selected model in nearly all cases included only the CO_2 treatment term without the random tank group term, therefore, tank group was dropped from the analysis. The greatest significant relative difference in size occured with width of the J6 stage, in which the crabs reared in ambient CO_2 were nearly 9% longer (supplemental Table 2). Crabs reared in ambient CO_2 had a wider, more oblong shape than crabs reared in high CO_2. There was no effect of CO_2 treatment on weight or on the quasi-density metric, weight/length. Sample sizes varied with crab abundance by stage and CO_2 treatment, with relatively low power at the J7 stage in all of the size metrics and in all but J5 for the weight metric.

The relationship between size and time (ignoring stage) was best described by the quadratic function relative to a linear model (AIC = 218). In the quadratic model, CO_2 treatment was highly significant ($p < 0.01$), with crabs reared in ambient CO_2 overall larger than those in high CO_2 (Fig. 8). Overall, the live carapace width correlated tightly with discarded molt exoskeleton, suggesting that molt size would be a reasonable proxy for crab size in future experiments (supplemental Fig. 7).

3.4. Respirometry

The respiration rate of juvenile crab reared and tested in high CO_2 was higher than the rate of crab reared in ambient CO_2 (mean ambient = 0.0963 mg O_2 g^{-1} h^{-1}; mean high = 0.0684 mg O_2 g^{-1} h^{-1}; $p = 0.002$; Fig. 9). Although there is no difference in the weight of crabs used in the respirometry analysis, there is a significant interaction between crab weight and treatment ($p = 0.015$). There is no relationship between weight and respiration rate for crabs reared in ambient CO_2 ($p = 0.277$), but there is a significant negative relationship between weight and respiration rate for crabs reared in high CO_2 (slope = −0.068; $p = 0.005$).



(caption on next column)

Fig. 2. Time series of the seawater chemistry of the juvenile exposure tanks. Panel A shows temperature values from durafet values in 5 of the experimental tanks (3 high-CO2 tanks and 2 ambient tanks). Each colour in Panel A indicates an individual experimental tank. Note that one of the ambient tank sensors stopped functioning on 12/19/18, so only 4 sensors are included after this date. Panel B shows the salinity(psu) measurements of two different sensors. The blue triangles show the average salinity value from all 6 experimental tanks using a hand-held Orion salinity sensor. The red circles represent daily salinity readings from an ATI toroidal salinity sensor in one of the experimental tanks. The ATI sensor did not come online until 11/20/18. Panel C shows the alkalinity values calculated from a Mukilteo specific alkalinity salinity curve (Trigg et al., 2019) from- either the Orion (blue triangles) or ATI (red circles) salinity sensors. Panel D shows spectrophotometric pH(Total) measurements from each of the treatment tanks. The ambient treatment tank symbols are colored red, green and ugly green and the high-CO_2 tank symbols are colored blue, light green and orange. Linear regression lines with 95% confidence intervals are shown separately for the ambient (magenta) and high-CO_2 treatments (redish pink). Note that spectrophotometric pH data were not recorded in January because of a U.S. Government shutdown, but data from the durafet pH sensors in the experimental tanks (not shown) indicate that the linear interpolation is reasonable. Panel E shows calcite saturation state (omega) calculated using SeaCarb (Gattuso et al., 2021) with input from the spectrophotometric pH, Orion salinity probe and alkalinity calculated from the Orion salinity probe. Panel E has the same gap in January because of the lack of pH data needed for the saturation state calculation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Dungeness crab megalopae exoskeleton condition has been suggested as a biological indicator of OA based on correlations with carbonate chemistry (e.g., $\Delta\Omega_{cal,60}$) across sites in the field (Bednarsek et al., 2020). In this experiment, megalopae had overall very high survival with no observed effect of CO_2 treatment on survival or development rate despite exposure to high CO_2 concentrations relative to future projections. This result suggests that megalopae are robust to high CO_2 concentrations and that changes in megalopae *abundance* would not be a sensitive biological indicator of ocean acidification. However, because Dungeness crab megalopae caught in light traps tend to be developmentally ready for rapid transition to the juvenile stage, the megalopae in our experiment experienced a short exposure time (mean < 4.3d) and their response to longer exposures is unknown. Although the experimental megolopae exposure was relatively short compared to the duration of the megalope stage in the wild (25–45 d) (Moloney et al., 1994), the experiment could have detected any acute effects of high CO_2 on survival or molting rate. A recent study found no relationship between the relative CO_2 sensitivity of zooplankton species groups measured in laboratory experiments and the correlation of the relative abundance of those same species groups with CO_2 gradients in the field (Keil et al., 2021), which highlights the potential for a mismatch between lab studies and field observations. Further work is needed to evaluate the utility of Dungeness crab megalopae as a biological indicator of OA.

For juvenile stage crabs, the result of higher crab survival when reared in higher CO_2 was surprising. The general pattern for most species, including crabs, is for lower survival in high CO_2 (Busch and McElhany, 2016; Kroeker et al., 2013; Wittmann and Pörtner, 2013). The higher survival of juvenile Dungeness crabs in high CO_2 contrasts with the larger size of crabs in ambient CO_2, suggesting a potential uncoupling of processes related to survival and growth. To explain the observation that more crabs live in high CO_2 but the surviving crabs are larger in ambient CO_2, we propose that there are two distinct processes affected by CO_2: one affects mortality and the other growth. The process driving the higher metabolic rate in high CO_2 may be the same as the survival or growth process or it could be the result of a third mechanism.

We suggest three alternative hypotheses for the process driving higher survival in high CO_2. The first hypothesis is the presence of an

Table 1
CO_2 exposure experiment sample sizes showing the number of Dungeness crab that molted into each stage.

Stage	High CO_2	Low CO_2	Total Count
Meg (initial wild)	123	99	222
J1	118	95	213
J2	79	55	134
J3	70	46	116
J4	65	42	107
J5	60	35	95
J6	50	15	65
J7	27	4	31

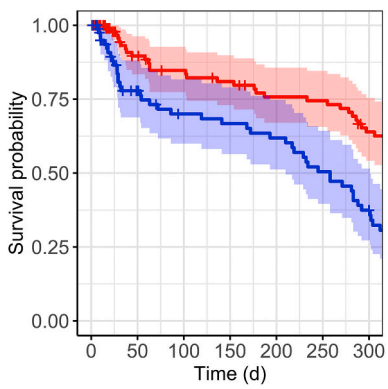


Fig. 3. Probability of survival for juvenile Dungeness crab reared in ambient CO_2 water (pH = 7.77; blue line) or high CO_2 (pH = 7.15; red line). The shaded areas show 95% confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

undetected pathogen or parasite that survives in low CO_2 but not high CO_2. Under this hypothesis, crabs susceptible to the pathogen would die at a higher rate in the low CO_2 treatment where the pathogen was present. Although the experiment was conducted in flow-through aquaria with high and low CO_2 treatment tanks being fed from a common head tank, the opportunity for different microbial or fungal communities to develop in each of the tanks existed since biofilms on the jars and tank walls could serve as a constant source of reinfection. Experiments have demonstrated the potential for dramatic shifts in biofilm communities in response to seawater pH (Nelson et al., 2020; Witt et al., 2011) and microbial abundance can decline in high CO_2 conditions (O'Brien et al., 2016). Parasites of the Eastern oyster are observed to decline under very low pH (Clements et al., 2017) and viruses are less abundant in low pH along a natural CO_2 gradient (Tangherlini et al., 2021). Under the cryptic pathogen hypothesis, crabs that survive the pathogen would be subject to largely non-lethal CO_2 effects on size and metabolic rate. Seawater delivered to the exposure tanks was pumped directly from Puget Sound and passed only through a coarse sand filter, which would not have removed any microbial pathogens. Crabs are vulnerable to a number of known pathogens (Morado, 2011; Wang, 2011), with several known to infect Dungeness crab (Armstrong et al., 1976, 1981; Cain and Morado, 2001; Childers et al., 1996; Morado et al., 1999; Sparks et al., 1982, 1985). There are undoubtedly numerous unidentified Dungeness crab pathogens and juveniles may be the most vulnerable stage in crustaceans (Behringer, 2012). Counter to the cryptic pathogen hypothesis is the observation that crabs (Holman et al., 2004; Meseck et al., 2016) and other species (Cao et al., 2018) can have reduced immune response at high CO_2. Since the crabs were reared in water pumped directly from Puget Sound, any pathogens present would be naturally occurring and, if this mechanism is operating, the high survival in high CO_2 observed in our experiment may occur in some places at some times in the wild. Although there is an overall effect of CO_2 on juvenile survival throughout the 300+ days of exposure, the survival effect is concentrated in the first juvenile stage. Under the

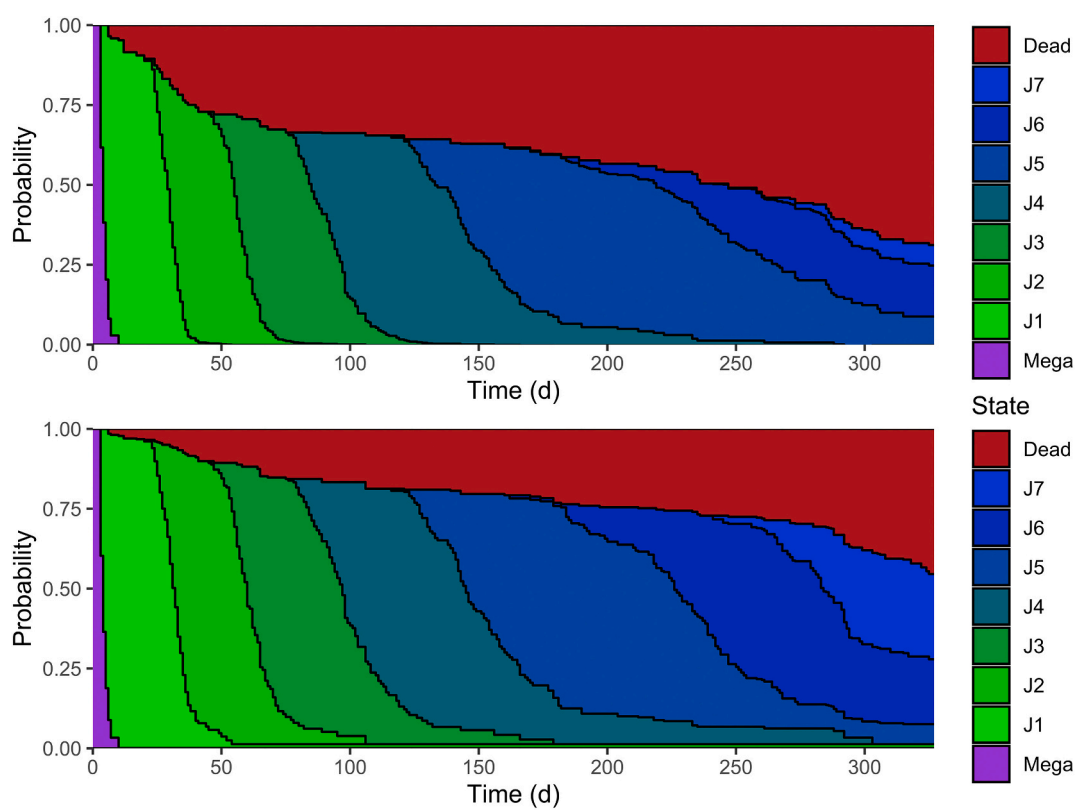


Fig. 4. Probability of Dungeness crab being in a given life-stage or dead as a function of time for ambient CO_2 (upper panel) or high CO_2 (lower panel).

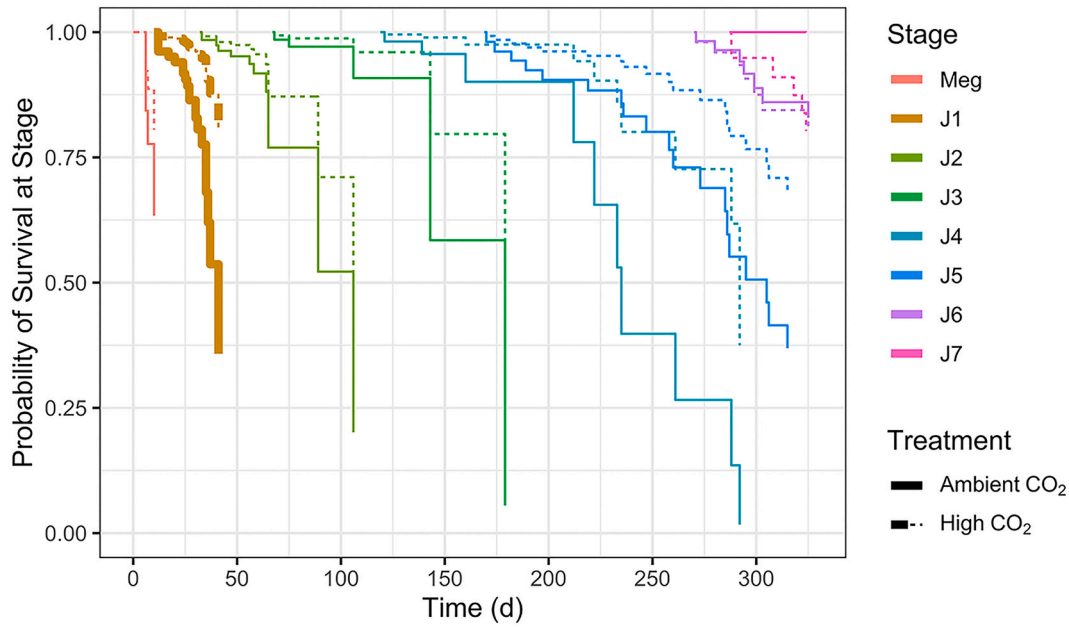


Fig. 5. Life-stage specific probability of survival as a function of time since the start of the experiment for Dungeness crab reared in ambient and high CO_2 water. The only life-stage with a statistically significant difference in survival as a function of CO2 treatment (J1) is indicated with a thicker line.

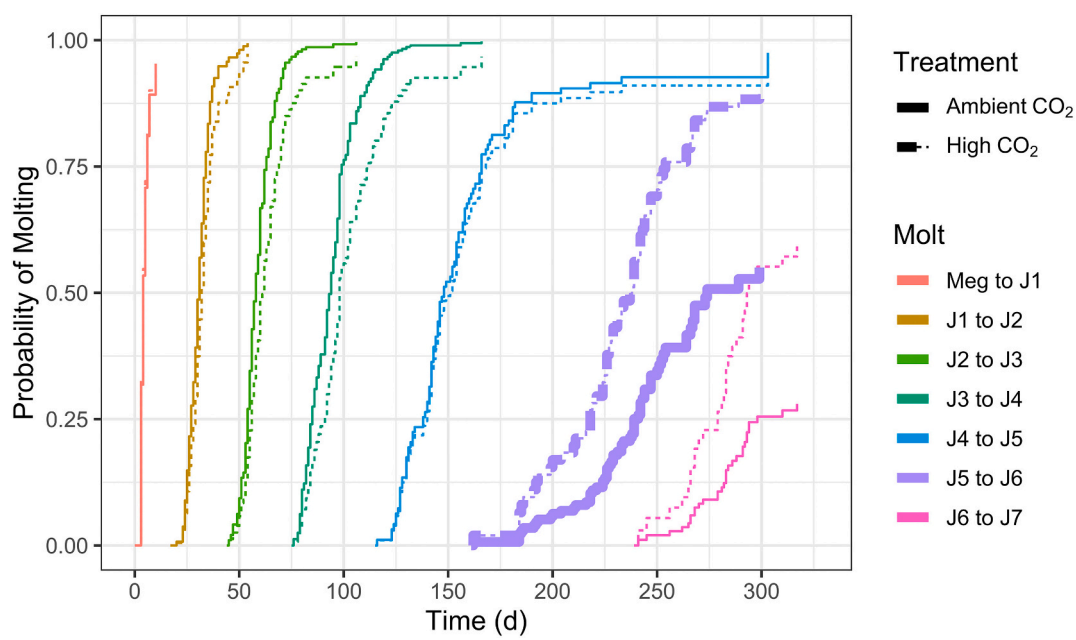


Fig. 6. Probability of molting between life-stages as a function of time since the start of the experiment for Dungeness crab reared in ambient and high CO_2 water. The only transition with a statistically significant difference in molting probability as a function of CO_2 treatment (J5 to J6) is indicated with a thicker line.

cryptic pathogen hypothesis, this indicates either 1) a heightened susceptibility at the earliest stage, 2) simply a "weeding out" of susceptible individuals on first encountering the pathogen, with the individuals remaining after stage 1 being largely resistant, or 3) an initial pulse of the pathogen which disappeared later in the experiment. We did not conduct pathogen tests of the crabs or water in this experiment so the cryptic pathogen hypothesis remains speculative. The complex interaction between host and pathogen/parasites in response to shifting CO_2 remains an understudied topic (MacLeod, 2017).

A second hypothesis is that Dungeness crab juveniles are adapted to high CO_2/low pH conditions. Our high CO_2 treatment (pH = 7.19) is in the range that can be encountered currently by juvenile Dungeness crab in some parts of the Salish Sea based on water column measurements (Feely et al., 2010; Greiner et al., 2018); the pH in the top layer of sediment where juvenile Dungeness crab hide may have even lower pH (Waldbusser and Salisbury, 2014). If Dungeness crab juveniles are adapted to this high CO_2 environment, they may survive less well when reared under lower CO_2. However, the observation that the juveniles in high CO_2 were smaller and had higher respiration rates suggests that high CO_2 had negative physiological impacts, which might lend support to the cryptic microbial pathogen hypothesis. Also, Dungeness crab juveniles are abundant in Puget Sound at locations where the CO_2 in the water column above them is similar to our ambient treatment – this high CO_2 adaptation hypothesis hinges on there being a difference between

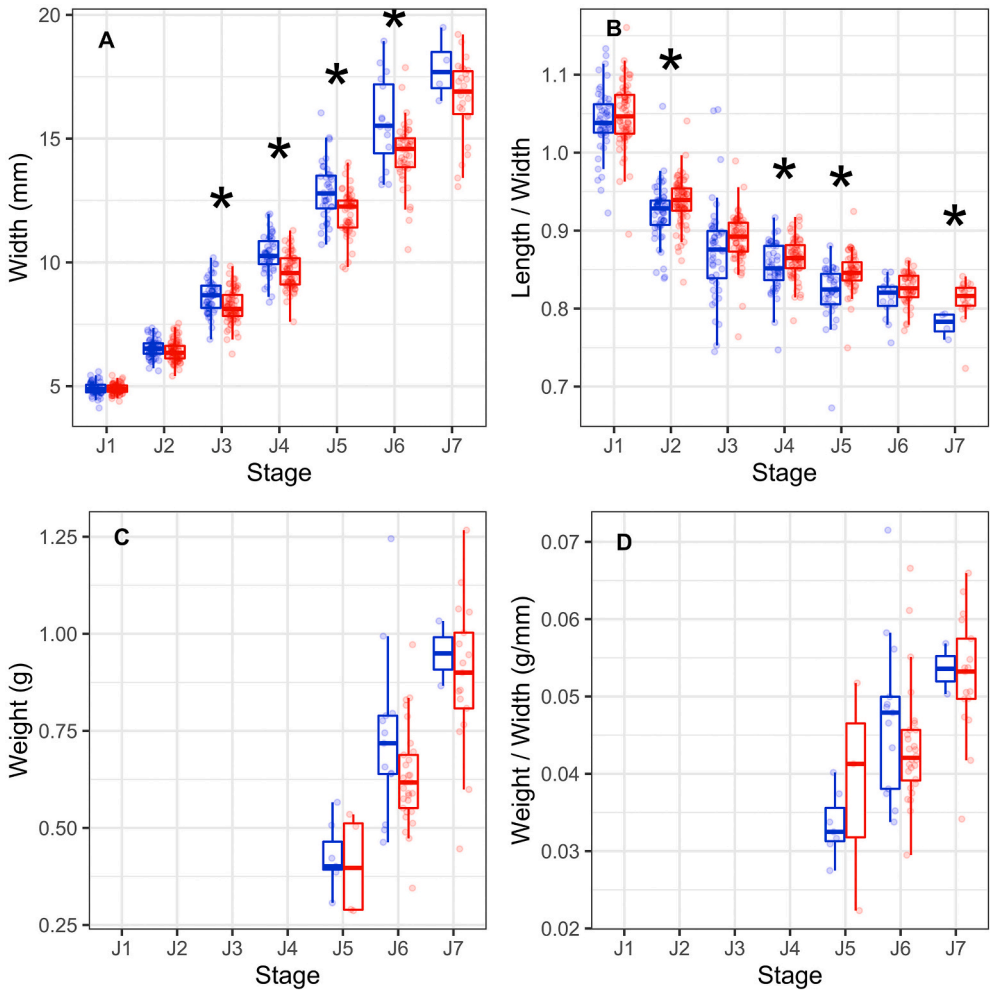


Fig. 7. Size measurements of Dungeness crab juveniles reared in ambient CO_2 (blue) and high CO_2 (red). Points show measurements for individual crabs, with the bar of the box plot indicating the median, the box showing 25th and 75th percentiles and the whiskers 1.5 * inter-quartile range. The black stars indicate juvenile stages with significant treatment differences. Width is a measure of carapace width, length/width is a ratio describing the shape of the crab, weight is live weight and weight/width is related to crab density. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Selected model and p-value results from size analysis of juvenile crabs reared in ambient and high CO_2 treatments. Significant *p*-values in bold.

Size metric	Selected Model	p-value (CO_2)	p-value (CO_2 x stage)
width	CO_2 + stage + CO_2 * stage + random(crab)	0.492	**<0.0001**
length	CO_2 + stage + random(crab)	**0.0201**	–
length/ width	CO_2 + stage + CO_2 * stage + random(crab) + random(tank)	0.4484	**0.0008**
weight	CO_2 + stage + CO_2 * stage	0.8648	0.7192
weight/ width	CO_2 + stage + CO_2 * stage	0.3921	0.3437

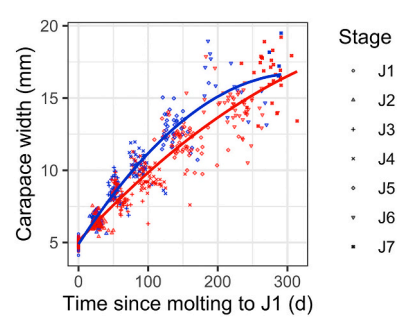


Fig. 8. Carapace width of Dungeness crab juveniles as a function of days since they molted to juveniles. Each point is an individual crab at a particular life-stage. Red indicates crabs reared in high CO_2 and blue indicates crabs reared in ambient CO_2. The curves show the fixed effects component of the repeated measures quadratic model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

water column CO_2 and the benthic microhabitat underneath rocks where juvenile crab are primarily found.

A third possibility is that there is a physiological trade-off between factors favoring reduced risk of early mortality and those favoring increased growth rate that played out in a complicated way during our experiment. The crabs reared in ambient conditions may have displayed a mortality risk vs. growth allocation strategy that is appropriate to conditions in the wild, where growing fast confers an advantage. Fast growth allows for escape from gape limited predators and makes it more likely an individual will on the winning side during cannibalistic interactions (Fernández, 1999). Crabs in high CO_2 on the other hand, may have expressed an early mortality risk vs. growth rate allocation strategy that appeared advantageous in the lab where predators are absent, but would be maladaptive in the wild. Why high CO_2 would trigger this shift in allocation is unclear. In particular, the higher survival observed in high CO_2 presumably has some non-trivial cost (otherwise, crabs in ambient CO_2 should match or exceed the survival rate of the high CO_2 crabs). Without a clear mechanism, this "shifting allocation hypothesis" also remains speculative.

There was little evidence for a large effect of CO_2 on developmental rate as indicated by a CO_2 treatment effect on molt timing in just one of seven observed stage transitions. The only significant effect of CO_2 on

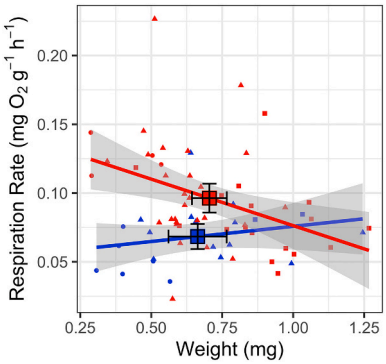


Fig. 9. Respiration rate of Dungeness crab juveniles from three different stages reared in ambient (blue) and high CO_2 (red) conditions as a function of weight. Each small point represents an individual crab (stage 5 = circles, stage 6 = triangle and stage 7 = small squares). The large squares indicate mean weight and respiration rate with error bars showing 95% confidence intervals on the means. The linear fits (solid lines) with 95% confidence intervals (grey transparent bands) show a significant relationship between respiration rate and weight for crabs in high CO_2 treatment but not those in ambient conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molt timing occurred when crabs in high CO_2 treatment had a shorter duration of the J5 stage than those in the ambient treatment. Again, this is somewhat counter-intuitive if high CO_2 is physiologically challenging. All else being equal, shorter intermolt intervals are likely advantageous as they indicate quicker development; previous studies have shown faster molting in ambient conditions for larval and juvenile crab (Ceballos-Osuna et al., 2013; Gravinese et al., 2018; Long et al., 2013; Miller et al., 2016; Schiffer et al., 2014; Walther et al., 2010). Although some stressors, such as loss of multiple limbs, may induce early molting, the phenomenon does not appear very wide-spread and the reverse stress response of delayed molting is much more common (Chang and Mykles, 2011). However, there may be energetic trade-offs between the size of each new molt and the frequency of molting, as has been hypothesized for molting patterns in American lobster (*Homarus americanus*) exposed to high CO_2 (McLean et al., 2018). Juveniles reared in high CO_2 were smaller at each stage than those reared in ambient CO_2 (Fig. 6) and crabs in high CO_2 may have molted quicker at the J5 stage to compensate for slower growth at earlier stages. Since crabs can only grow when they molt, molting faster may be an effort to "catch up" in size and, indeed, by the end of the experiment crabs in both treatments were approaching a similar size, ignoring stage (Fig. 7). The overall pattern of size difference between ambient and high-CO_2 crabs maybe the consequence of different feeding behaviors, allocation of energy to growth or maintaining acid-base balance or other physiological trade-offs. Dungeness crab juveniles are vulnerable to cannibalism and other size-selective predation, so there is selective pressure for adaptations to increase in size rapidly.

The base metabolic rate, as measured by oxygen uptake (respiration), increased in juveniles reared in high CO_2 which may be the result of an increase in energetic cost of maintaining internal acid-base balance. This mechanism has been invoked in the context of the oxygen and capacity limited thermal tolerance hypothesis (Pörtner, 2001, 2021; Portner, 2010), where the increase in metabolic rate at high CO_2 is hypothesized to drive a reduction the temperature range of anaerobic scope. Increased metabolic rate is consistent with a metabolomics analysis of Dungeness crab megalopae, which found an increase in metabolites associated with ATP production (Trigg et al., 2019). The cause of differential treatment effects of crab weight on base metabolic rate is less clear (Fig. 8). As crabs get larger, their respiration rate appears to become less sensitive to CO_2. This might occur if there are size-dependent shifts in the gill surface area to volume ratio.

Increased seawater CO_2 has shown mixed effects on resting metabolic rate, as measured by oxygen intake, in other crab species. In adult velvet swimming crab (*Necora puber*), increasing seawater CO_2 causes a decrease in base metabolic rate (Small et al., 2010). In one study of the porcelain crab, *Petrolisthes cinctipes*, embryos had lower oxygen intake at high CO_2 but CO_2 treatment had no effect on oxygen intake for larvae or juveniles (Carter et al., 2013). In another study of porcelain crab, larvae again expressed no effect of CO_2 alone on oxygen intake, but CO_2 treatment did show an interaction with subsequent exposure to stressful salinity conditions – only crabs reared at high CO_2 showed an increase in metabolic rate when later exposed to low or high salinity (Miller et al., 2014). Both juvenile red and blue king crab (*Paralithodes camtschaticus* and *P. platypus*) had a higher oxygen intake when initially introduced to high CO_2 conditions, but after 3 weeks acclimation there was no difference in oxygen intake among pH treatments (Long et al., 2020). As described in a meta-analysis of the respiratory effects of temperature and CO_2 on marine ectotherms (Lefevre, 2016), the mixed resting metabolic rate responses observed in crabs is common in other marine taxa – most species show no effect of CO_2 whereas some show an increase or decrease in respiration. Increases in metabolic rate, as we observe in Dungeness crab juveniles, is hypothesized to be driven by increased energetic costs of maintaining acid-base balance and/or calcification. The mechanism responsible for lower metabolic rate in high CO_2 conditions as observed in some species is less clear (Lefevre, 2016) – it may be a lowering of metabolic activity to temporarily conserve energy or perhaps, the anesthetizing effect of increased Mg in the haemolymph that is create as a by-product of maintaining internal acid-base balance (Spicer et al., 2007).

5. Conclusion

A primary goal of this study was to gain insight into how the Dungeness crab might respond at a population level to current and future variation in carbonate chemistry. Addressing this question requires an evaluation of multiple life-stages. Previous experiments show a decrease in survival of zoea at high CO_2 (Miller et al., 2016). The results of this study suggest that megalopae are relatively robust to a high CO_2 exposure, at least for exposures lasting several days. The response of juveniles is more complex, in that high CO_2 had a positive effect on survival, but a negative effect on growth. There is certainly no indication of acute or long-term increased mortality at high CO_2 – some juveniles survived for nearly a year in water with pH < 7.2. In fact, counter to expectation, crabs in this experiment survived better in high CO_2 than in ambient CO_2. Whether this higher survival in high CO_2 is the consequence of a cryptic pathogen, the result of underlying physiological optima at low pH, is caused by mortality risk vs. growth trade-offs, or the product of some other mechanism, has important implications for projecting population dynamics of Dungeness crab in an acidifying ocean. If the result is caused by a cryptic pathogen, higher survival would only occur under a select set of circumstances involving pathogen exposure and high CO_2 and therefore may have limited impact on population dynamics (i.e., CO_2 concentration would not have much effect on survival most of the time and the negative sublethal effects of CO_2 on growth and respiration would dominate the crab response). If the result was caused by survival vs. growth trade-offs that are unique to the laboratory setting, there might be a negative effect of high CO_2 on crab populations in wild because crabs in high CO_2 are smaller and therefore more vulnerable to predation. However, if the result is driven by physiological optima, increased CO_2 may have a positive effect on population dynamics, though it is important to note that this study did not evaluate the relative influence of CO_2 compared to other potential drivers such as temperature or prey availability (Marshall et al., 2017). A shortage of calcareous food sources combined with a higher energy demand in a high CO_2 environment could lead to food deprivation effects that were undetected in this experiment where food was supplied ad libitum. Other species show positive and negative effects of high CO_2 depending

on which specific response is measured (e.g. survival, growth, nutrition content, etc.) suggesting the importance of examining a range of biological metrics and for robust replication (Hurst et al., 2021). Although further work is required to understand the effect of elevated CO_2 on Dungeness crab megalopae and juveniles, research to date suggests that zoea are the most sensitive stage of this species, counter to observations in American lobster (Noisette et al., 2021).

CRediT authorship contribution statement

Paul McElhany: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Shallin Busch:** Conceptualization, Formal analysis, Writing – review & editing. **Amanda Lawrence:** Methodology. **Michael Maher:** Methodology, Investigation, Resources, Data curation. **Danielle Perez:** Investigation, Resources, Visualization, Data curation. **Emma Reinhardt:** Methodology, Investigation, Formal analysis. **Kate Rovinski:** Investigation, Resources, Data curation. **Erin Tully:** Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2022.151781.

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